

## Research Article

# Ex Vivo Stromal Cell-Derived Factor 1-Mediated Differentiation of Mouse Bone Marrow Mesenchymal Stem Cells into Hepatocytes Is Enhanced by Chinese Medicine Yiguanjian Drug-Containing Serum

Linlin Fu,<sup>1</sup> Bingyao Pang,<sup>1</sup> Ying Zhu,<sup>1</sup> Ling Wang,<sup>2</sup> Aijing Leng,<sup>3</sup> and Hailong Chen<sup>4</sup>

<sup>1</sup>Department of Infectious Disease, The First Affiliated Hospital of Dalian Medical University, No. 222, Zhongshan Road, Xigang District, Dalian 116011, China

<sup>2</sup>Department of Digestive Disease, Gansu Provincial Hospital, Lanzhou 730000, China

<sup>3</sup>Department of Chinese Medicine, The First Affiliated Hospital of Dalian Medical University, Dalian 116011, China

<sup>4</sup>Department of General Surgery, The First Affiliated Hospital of Dalian Medical University, Dalian 116011, China

Correspondence should be addressed to Ying Zhu; zhuyingsh52@126.com and Hailong Chen; hailongchen2007@hotmail.com

Received 29 December 2015; Revised 4 March 2016; Accepted 23 March 2016

Academic Editor: Yuewen Gong

Copyright © 2016 Linlin Fu et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Yiguanjian is administered in traditional Chinese medicine for liver diseases and has been demonstrated to reduce liver fibrosis. This study investigated the effect of Yiguanjian drug-containing serum (YGJ) with Stromal Cell-Derived Factor 1 (SDF-1) and Hepatocyte Growth Factor (HGF) on the differentiation of murine bone-marrow-derived mesenchymal cells (BM-MSCs) into hepatocytes *in vitro*. Adherent MSCs were isolated from murine bone marrow. Differentiation was induced by 20 ng/mL HGF, 50 ng/mL SDF-1, and 20% Yiguanjian drug-containing serum for 7 to 28 days, and mature hepatocytes' marker albumin (ALB) and cholangiocytes' marker cytokeratin-18 (CK-18) were assessed by immunocytochemistry and western blot. BM-MSCs exhibited homogeneous spindle shape growth after subculture and stained positive for CD90 and negative for CD34. After induction with HGF + normal serum or YGJ for 14 days, HGF + SDF-1 + normal serum for 7 days, or HGF + SDF-1 + YGJ for 5 days, MSCs' morphology changed gradually and begun to resemble hepatocyte-like cells. Cultures supplemented with HGF + SDF-1 + YGJ contained significantly higher proportions of ALB and CK-18 positive cells than cultures supplemented with HGF + SDF-1 + normal serum at day 7. These observations corroborated the results of western blot. In conclusion, Yiguanjian drug-containing serum could facilitate the differentiation of murine BM-MSCs into hepatocytes *in vitro* and has a synergistic effect with SDF-1 and HGF.

## 1. Introduction

Liver transplantation is the most effective therapy for the patients with advanced liver diseases. However, the availability of donor livers limits application of this therapy [1, 2]. Recently, stem cell-based cytotherapy has been demonstrated to benefit some patients with liver disease [3–11].

BM-MSCs are relatively easy to separate and collect, exhibiting a relatively stable genetic background and extremely strong proliferative capacity and substantial plasticity and being capable of differentiation into liver parenchyma cells, liver sinus endothelial cells, Kupffer cells,

stellate cells, and muscle fibroblasts [12–14]. Autologous BM-derived MSCs also induce only weakly immune rejection, and their use does not involve any complicated ethical or moral questions, recommending these cells for stem cell transplantation in the treatment of end-stage liver disease [12].

BM-MSCs can be readily isolated from bone marrow samples but represent a heterogeneous group, and responses in the clinic can vary accordingly [12]. Specific surface markers for MSCs are yet to be defined, limiting the capacity to isolate this population, and although effective transplantation likely requires large numbers of cells, *ex vivo* expansion

methods which encourage differentiation into hepatocytes have not yet been perfected [3–11].

Stem cell microenvironment is a decisive factor to the differentiation specific of stem cells. Supplementing BM-derived MSC culture with HGF promoted mitosis by interaction with receptor c-met, enhancing migration capacity and promoting mesoderm and ectoderm-derived cell proliferation, thereby promoting cell mitosis and morphogenesis and directing differentiation into hepatocytes [15–18]. SDF-1, also known as pre-B cell stimulating factor (PBSF) or CXCL12, is a widely expressed chemokine differing by just one amino acid in human and murine forms. MSCs *in vitro* have been reported to express the SDF-1 receptor, CXCR4, and signaling induces MSC chemotaxis and homing to the liver [19–21]. We sought to further enhance this *ex vivo* culture environment to encourage proliferation of BM-derived MSC and direct differentiation towards hepatocytes.

The traditional Chinese medicine Yiguanjian (described in the “Liuzhou Medical Talks”) is traditionally administered for liver diseases. It is comprised of radix glehniae, radix ophiopogonis, radix *Angelicae sinensis*, dried rehmannia root, *Lycium barbarum* L., and fructus meliae toosendan. This formula has a high concentration of polysaccharides, as each of *Lycium barbarum* L., radix *Angelicae sinensis*, radix ophiopogonis, and radix glehniae contains polysaccharides. There are 18 kinds of amino acids in this decoction, of which there are eight kinds of essential amino acids for humans. And it also contains many trace elements, microelements, saponins, phytosterols, triterpenoids, lactones, coumarins, and flavonoids. There are many researches indicating that, in both rat and mouse models of liver disease, Yiguanjian could reduce liver fibrosis [22–26], and oral administration of Yiguanjian decoction significantly reduced the serum aspartate aminotransferase (AST) and alanine transaminase (ALT) and inhibited accumulation of collagen I, tissue inhibitor of metalloproteinase-1, and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) in hepatic tissues. Yiguanjian improves liver function in rats, reducing histological damage and increased expression of hepatic oval cells. Wang et al. [26] revealed that Yiguanjian inhibited liver fibrogenesis by inhibiting bone marrow cells differentiating into myofibroblasts in the liver. Hence, we sought to determine whether supplementing cultures with Yiguanjian drug-containing serum *ex vivo* could enhance BM-MSCs’ differentiation towards hepatocytes and cholangiocytes in isolated mouse BM-MSCs.

## 2. Materials and Methods

**2.1. Animals.** Male Kunming mice ( $n = 140$ , body weight  $18 \pm 2$  g) aged 4–5 weeks were obtained from the specific-pathogen-free (SPF) level animal experimental Center of Dalian Medical University (China) (license number: SCXK (Liao) 2008-0002). Animals were housed at 20–25°C and  $50 \pm 5\%$  humidity with ad libitum access to food and water and a 12:12 h light/dark cycle. All procedures and animal experiments were approved by the Animal Care and Use Committee of Dalian Medical University (China). The mice were prepared for isolation of BM-MSCs and preparation of Yiguanjian drug-containing serum.

**2.2. Preparation of Yiguanjian Decoction.** A decocted concentrated liquid of Yiguanjian was produced using radix glehniae 9 g, radix ophiopogonis 9 g, radix *Angelicae sinensis* 9 g, dried rehmannia root 18 g, *Lycium barbarum* L. 9 g, and fructus meliae toosendan 5 g at the Chinese Medicine Center of the First Affiliated Hospital of Dalian Medical University. The decoction was prepared according to the original proportion and preparation method; then the filtrate was concentrated and dried into powder, and 1 g of the extract contained 2.3 g herbs.

**2.3. Preparation of Yiguanjian Drug-Containing Serum.** Mice were divided into two groups ( $n = 50$  each group): normal control group and Yiguanjian decoction-treated group. 50 mice received Yiguanjian decoction in the dose of 0.016 mL/g body weight/day by gavage twice (with an interval of more than 6 hours) every day for 3 days. The daily dose is 10-fold as that of 60 kg adult, which is 10 mL/kg body weight. Animals in the normal control group received an equivalent volume of normal saline (NS). One hour after intragastric administration on the third day, blood was sampled from the eyeball, stored at 4°C for 4 hours, and then centrifuged at 1509 g for 20 min. The supernatant serum was mixed, sterilized and inactivated at 56°C for 30 min, and stored at –70°C.

**2.4. Isolation and Culture of BM-MSCs.** BM-MSCs were isolated from bone marrow, as previously described [27, 28]. Mice ( $n = 40$ ) were sacrificed by cervical dislocation. The femur and tibia were removed and soaked in 75% alcohol for 3 min; then the bone marrow cavity was rinsed with DMEM/F 12 medium (HyClone). Bone marrow fluid was centrifuged at 377 g for 10 min, and the supernatant was discarded. Cells were resuspended at  $1 \times 10^9$  cells/L in DMEM/F 12 medium containing 15% FBS (Gibco) and 100 U/mL penicillin-streptomycin and incubated at 37°C at an atmosphere of 5% CO<sub>2</sub>. After 48 hours the culture medium was changed, and nonadherent hematopoietic cells were removed. The medium was then changed every 3–4 days, and cells were observed by Eclipse TS100 inverted microscopy (Nikon, Japan). When cells grew to 80–90% confluency (about 12–14 days), the monolayer was digested with 0.25% trypsin (Hyclone) containing 0.02% EDTA, and cells were passaged at the dilution of 1:2 for one to three rounds (P1 to P3).

**2.5. Identification of BM-MSCs.** CD90 expression in passage-two MSCs was determined by immunocytochemistry (rabbit anti-mouse CD90 and SP immunohistochemistry kit from Bioss Biotechnology Co., Ltd., Beijing, China), according to the manufacturer’s instructions. Color was developed with 3,3'-diaminobenzidine (DAB) concentration reagent kit (Rope Lai Valuable Company, Beijing) by incubating at room temperature for 5 minutes in dark. Then coloration was finally finished by hematoxylin staining. The slides were observed under the Leica DMIL-LED inversion phase contrast microscope.

After two passages, 100  $\mu$ L BM-MSCs ( $1 \times 10^7$  cells/mL) were stained with fluorescein isothiocyanate- (FITC-) labeled rabbit anti-mouse CD90 (eBioscience, San Diego, CA) or

PE-labeled rabbit anti-mouse CD34 (a BioLegend product, San Diego, CA) for 30 min at 4°C in the dark. Flow cytometry analysis was performed by FACS Vantage Flow Cytometer with CellQuest software (Becton Dickinson, USA).

**2.6. Measurement of BM-MSCs' Growth.** Second-passage BM-MSCs in the logarithmic growth phase were resuspended at  $5 \times 10^3$ /mL and 200  $\mu$ L was plated in a 96-well plate. Blank wells were only medium without cells. The viability of cells was determined by the MTT method each day for 7 days. Briefly, 20  $\mu$ L MTT solution (5 mg/mL, Ameresco, USA) was added to each well and incubated at 37°C with 5% CO<sub>2</sub> for 4 hours before the supernatant was replaced with 150  $\mu$ L DMSO (Sigma, USA). After shaking for 10 min, the OD at 490 nm was measured on a microplate reader (Thermo, USA). This process was repeated for 3 times.

**2.7. Induction of BM-MSCs' Differentiation In Vitro.** Second-passage MSCs ( $4 \times 10^5$  cells/well) in the logarithmic phase of growth were cultured in 6-well plates. When having reached 70–90% confluency, cells were incubated with the following mediums for 7, 14, 21, and 28 days. Negative control cells were cultured in medium containing 15% normal serum, but cells at HGF + normal serum group (HGF group) received medium supplemented with a final concentration of 20 ng/mL HGF (Peprotech, USA) and 15% normal serum (as previously established [29, 30]). And cells at Yiguanjian drug-containing serum group (YGJ group) received medium supplemented with 20% Yiguanjian drug-containing serum. Cells at HGF + SDF-1 + normal serum group (HGF + SDF-1 group) received medium supplemented with a final concentration of 20 ng/mL HGF + 50 ng/mL SDF-1 (Peprotech, USA) and 15% normal serum (as previously established [31, 32]). Cells at HGF + SDF-1 + Yiguanjian drug-containing serum group (HGF + SDF + YGJ group) received medium supplemented with a final concentration of 20 ng/mL HGF + 50 ng/mL SDF-1 + 20% Yiguanjian drug-containing serum.

### 2.8. Characterization of Cells Differentiated from BM-Derived MSCs In Vitro

**Morphological Observation.** BM-derived MSC cultures were observed daily by inverted microscope. To assess differentiation, markers of hepatocyte-like differentiation, cellular ALB, and CK-18 expressions were assessed on 7, 14, 21, and 28 days after induction by immunocytochemistry and western blot.

For immunocytochemistry, cells were incubated with the following antibodies: rabbit anti-mouse ALB (1:200), rabbit anti-mouse CK-18 (1:200) (Bioss Biotechnology Co., Ltd., Beijing, China) overnight at 4°C followed by DAB staining. The numbers of total cells and ALB or CK-18 positive cells were counted in five randomly selected fields by three independent investigators blinded to grouping, and the percentage of ALB and CK-18 positive cells was calculated.

For western blot analysis, total protein was extracted in ice-cold lysis buffer (Beyotime, Haimen, China) containing proteinase inhibitor PMSF (KeyGEN BioTECH, Nanjing, China). The lysate were subjected to 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore,

Bedford, MA). The membranes were blocked and then incubated with anti-mouse ALB (1:2000) (Proteintech) and anti-mouse CK-18 (1:500) (Proteintech) overnight at 4°C. After washing, the membranes were incubated with horseradish peroxidase-conjugated IgG (diluted 1:10000, Bioss) at 37°C for 1 hour. The immunoreactive bands were detected by ECL system (Advansta, USA) and analyzed by AlphaView software (ProteinSimple, USA).

**2.9. Statistical Analysis.** SPSS13.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis, data are shown as mean  $\pm$  standard deviation (SD), and comparisons among groups were performed using one-way analysis of variance (ANOVA) with *q* test for *post hoc* analysis. *P* < 0.05 was considered statistically significant.

## 3. Results

**3.1. Characterization of Murine BM-MSCs.** Adherent cells were isolated from murine bone marrow and passaged *in vitro*, termed BM-MSCs. Four hours after isolation, BM-MSCs began to adhere to the cell culture flask, and within 24 h the majority of cells were adhered (Figure 1(a)). During prolonged incubation, the cells aggregated into clusters, and with the exception that few cells were polygonal or round in shape, most cells were spindle-shaped (Figure 1(b)). MSCs tended gradually to confluency after growth of 12–14 days (Figure 1(c)). The MSCs were subcultured by trypsin digestion when the density reached 70% to 80%. After subculture, the MSCs refraction was better and spindle shape growth was more uniform (Figures 1(d), 1(e), and 1(f)).

The proliferation capacity of BM-MSCs (P3) was measured by MTT assay. While cells grew slowly within the first 2 days of culture, the number of cells increased between days 4 and 6 and then plateaued until day 7 (Figure 1(g)).

To determine the nature of the isolated cells (P2), they were stained for CD34 and CD90. Cells were positive for CD90 and negative for CD34 (Figure 2).

**3.2. Morphology Change of BM-Derived MSCs during Differentiation.** BM-MSCs (P2) cultured with 15% normal serum were spindle-shaped, spiral-shaped, or whirlpool-shaped (Figure 3(a)). After induction with 20 ng/mL HGF + 15% normal serum for 14 days or 20 ng/mL HGF + 50 ng/mL SDF-1 + 15% normal serum for 7 days, BM-MSCs proliferation gradually reduced, and cells clustered, projections retracted, and cell morphologically changed from irregular polygonal to large, round, less-adherent cells with clear nuclei and abundant cytoplasm, partially resembling hepatocyte-like cells (Figures 3(b) and 3(c)). Cells cultured with 20 ng/mL HGF, 50 ng/mL SDF-1, and 20% Yiguanjian drug-containing serum for 5 days were also circular, partially resembling hepatocyte-like cells (Figure 3(d)). After induction with 20% Yiguanjian drug-containing serum for 14 days, morphology of cells began to change (Figure 3(e)).

**3.3. ALB Protein Level during Differentiation of BM-Derived MSCs.** Immunocytochemical staining for ALB was observed

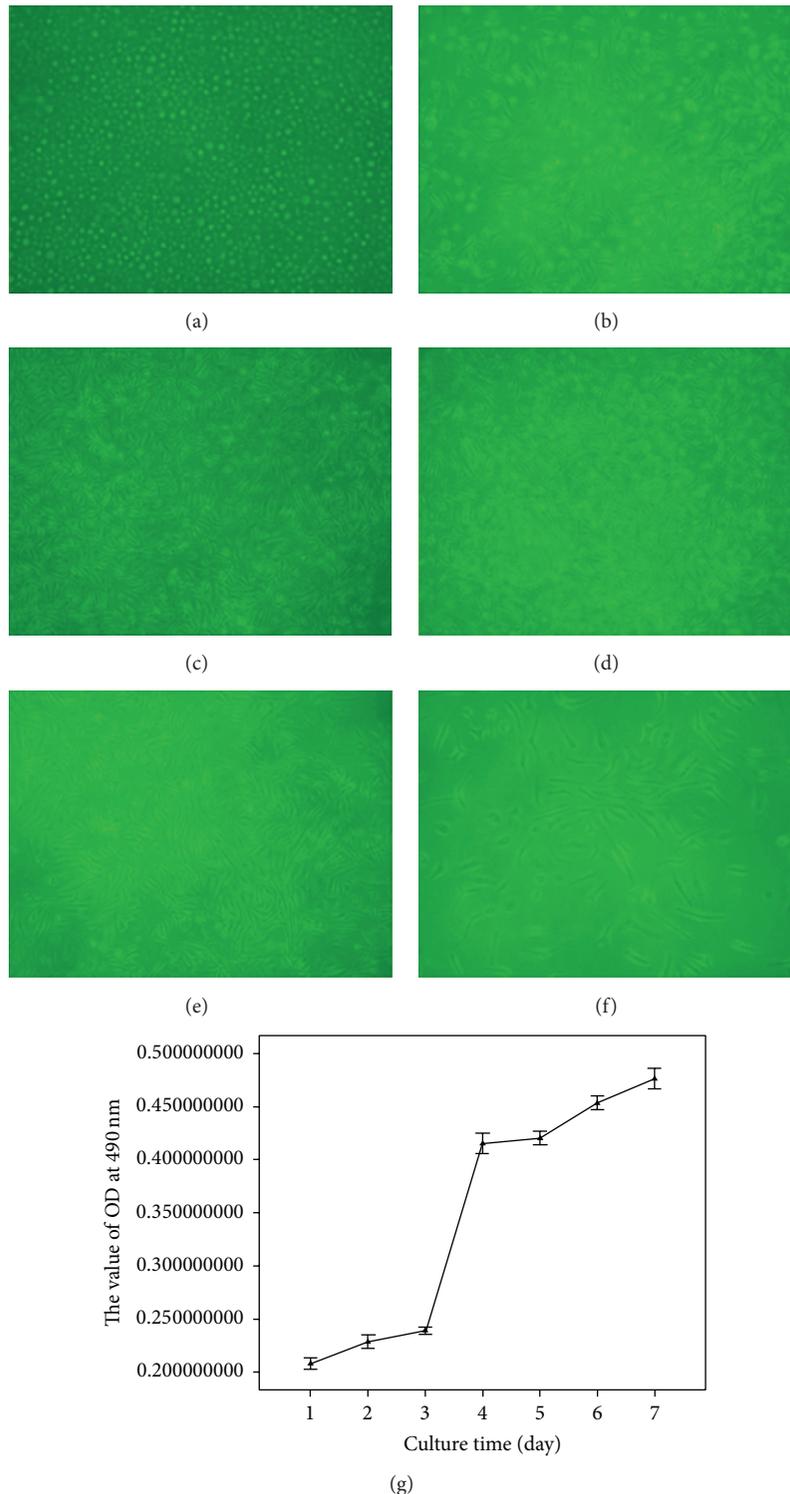


FIGURE 1: Morphology and growth of mouse bone-marrow- (BM-) derived Mesenchymal Stem Cells (MSCs). (a) BM-MSCs were isolated based on adherence and cultured for 24 hours. (b) Primary MSCs cultured for 7 days; the cells showed colony-like growth, shuttle shape with synapses, large and clear nuclei, and abundant cytoplasm. (c) Primary MSCs cultured for 15 days; cells gradually became fused. (d) MSCs at first passage (P1) cultured after 7 days; cells diffraction was better. (e) MSCs at second passage (P2) cultured after 7 days. (f) MSCs at third generation (P3) cultured after 7 days; P1, P2, and P3 MSCs were all in homogeneous spindle shape growth (magnification:  $\times 10$ ). (g) BM-derived MSCs at P3 grew slowly at 1-2 days; cells were in a logarithmic growth phase at 3-4 days; and cells entered a platform phase after 7 days.

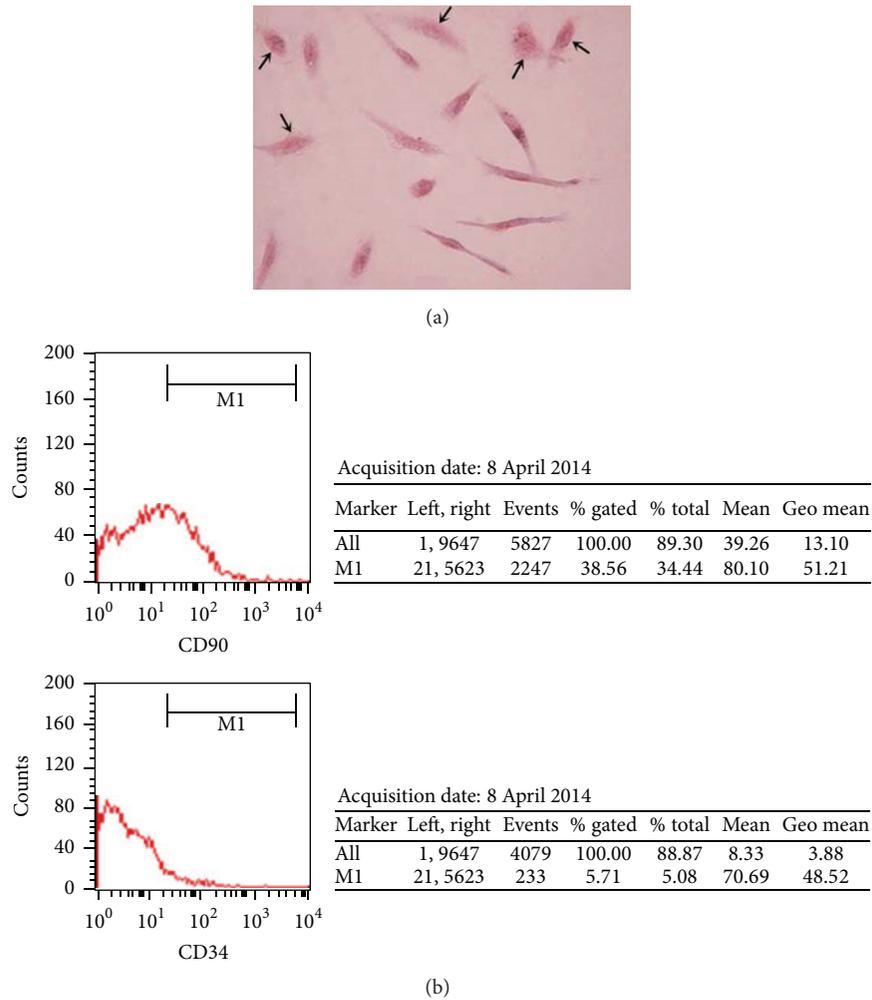


FIGURE 2: Characterization of BM-derived MSCs surface expression of CD90 and CD34. (a) CD90 expression at P3 MSCs determined by immunocytochemical staining (ICC) (magnification:  $\times 40$ ). Black arrows indicate MSCs positively stained for CD90 (brown staining, most obvious around the nuclei). (b) BM-MSCs' nature of these cells (P2) was confirmed based on positivity for CD90 (38.56%) and negativity for CD34 (5.71%).

at day 14 in cells cultured with 20 ng/mL HGF + 15% normal serum, and positive staining intensity increased until day 28 (Figure 4(a)). ALB positive staining was observed earlier in cells cultured with 20 ng/mL HGF + 50 ng/mL SDF-1 + 15% normal serum or those cultured with 20 ng/mL HGF + 50 ng/mL SDF-1 + 20% Yiguanjian drug-containing serum, and positive staining intensity increased from day 7 until day 28 (Figures 4(b) and 4(c)). And ALB positive staining cells in YGJ group could be observed at day 14 after induction and increased gradually to day 21, and there was a reduction at day 28 (Figure 4(d)). Cultures supplemented with HGF, SDF-1, and Yiguanjian drug-containing serum contained a significantly higher proportion of ALB positive cells than cultures supplemented with HGF, SDF-1, and normal serum at 7 ( $51.96 \pm 3.17\%$  versus  $19.27 \pm 1.91\%$ ), 14 ( $65.53 \pm 2.02\%$  versus  $47.22 \pm 1.62\%$ ), and 21 days ( $72.95 \pm 0.95\%$  and  $55.80 \pm 1.03\%$ ) (all  $P < 0.05$ ), indicating that ALB expression appeared more rapidly in the former cultures (Table 1). However, by day 28, there was no significant difference in

the fraction of ALB positive cells in cultures supplemented with HGF, SDF-1, and normal serum or HGF, SDF-1, and Yiguanjian drug-containing serum (Table 1).

These observations corroborated the results of western blot quantification; ALB protein expression sharply increased at day 28 in cultures supplemented with HGF and normal serum (Table 2 and Figure 5(a)); however, ALB protein expression progressively increases from day 7 to day 28 in cultures supplemented with HGF, SDF-1, and normal serum, and protein expression at day 7 was significantly higher than that in cultures supplemented with HGF and normal serum (Table 2 and Figure 5(b)). ALB protein expression in cultures supplemented with HGF, SDF-1, and Yiguanjian drug-containing serum was higher than those in cultures supplemented with HGF, SDF-1, and normal serum at days 14 and 21 (Table 2 and Figure 5(c)). Expression of ALB increased from day 14 to 21 in culture supplemented with Yiguanjian drug-containing serum (Table 2 and Figure 5(d)).

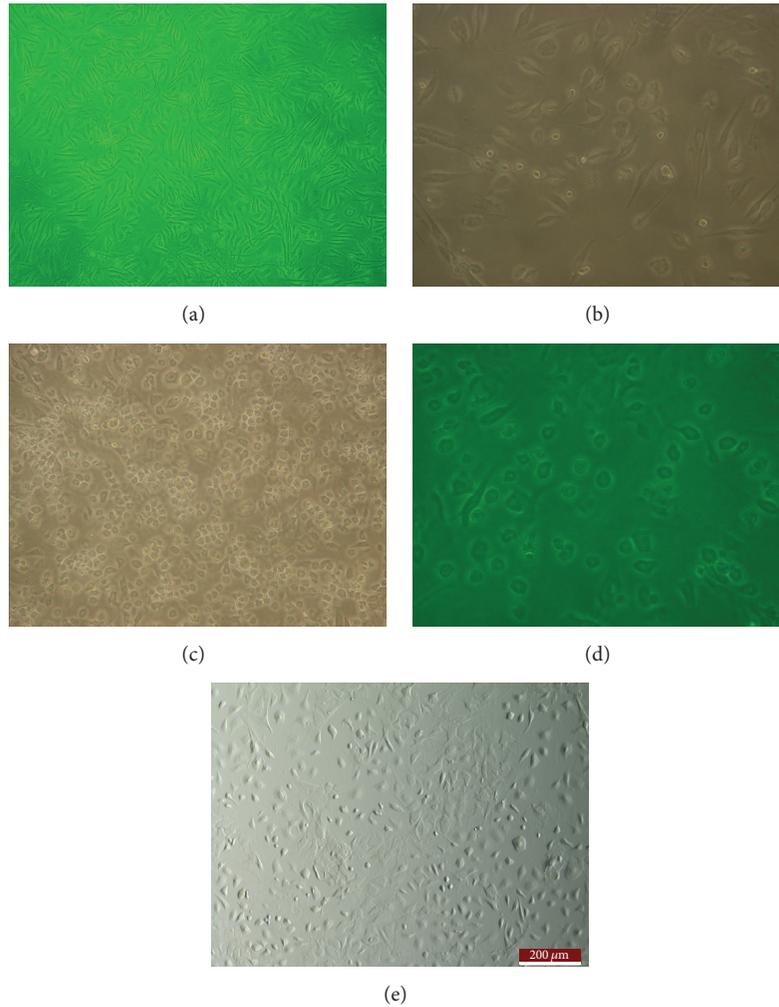


FIGURE 3: Differentiated cell morphology from P2 BM-derived MSCs. BM-derived MSCs (P2) were cultured with 15% normal serum (magnification:  $\times 10$ ) and were spindle-shaped, spiral-shaped, or whirlpool-shaped (a). MSCs cultured with 20 ng/mL HGF + 15% normal serum for 14 days (magnification:  $\times 20$ ) became polygonal and circular, partially resembling hepatocyte-like cells (b). MSCs cultured 20 ng/mL HGF + 50 ng/mL SDF-1 + 15% normal serum for 7 days (magnification:  $\times 20$ ) were polygonal and circular, partially resembling hepatocyte-like cells (c). MSCs cultured with 20 ng/mL HGF + 50 ng/mL SDF-1 + 20% Yiguanjian drug-containing serum for 5 days (magnification:  $\times 20$ ) were polygonal and circular, partially resembling hepatocyte-like cells (d). MSCs cultured with 20% Yiguanjian drug-containing serum for 14 days (magnification:  $\times 10$ ) (e).

TABLE 1: Albumin (ALB) in second-passage (P2) bone marrow- (BM-) derived Mesenchymal Stem Cells (MSCs) determined by immunocytochemical staining (ICC).

	ALB <sup>+</sup> BM-derived MSCs (%)			
	7 days	14 days	21 days	28 days
Negative controls	0	0	0	0
HGF + normal serum	0	24.96 $\pm$ 2.76	36.18 $\pm$ 1.45	67.15 $\pm$ 2.78 <sup>△</sup>
HGF + SDF-1 + normal serum	19.27 $\pm$ 1.91	47.22 $\pm$ 1.62	55.80 $\pm$ 1.03	71.03 $\pm$ 1.21 <sup>△</sup>
HGF + SDF-1 + YGJ	51.96 $\pm$ 3.17 <sup>*</sup>	65.53 $\pm$ 2.02 <sup>*</sup>	72.95 $\pm$ 0.95 <sup>*</sup>	76.84 $\pm$ 1.70
YGJ	8.06 $\pm$ 0.59	13.33 $\pm$ 1.75 <sup>#</sup>	21.43 $\pm$ 1.08 <sup>#</sup>	16.36 $\pm$ 2.68

Note. Negative controls: MSCs were cultured in medium containing 15% normal serum; HGF + normal serum: MSCs were cultured in medium supplemented with a final concentration of 20 ng/mL HGF and 15% normal serum; HGF + SDF-1 + normal serum: MSCs were cultured in medium supplemented with a final concentration of 20 ng/mL HGF + 50 ng/mL SDF-1 and 15% normal serum; HGF + SDF-1 + Yiguanjian drug serum: MSCs were cultured in medium supplemented with a final concentration of 20 ng/mL HGF + 50 ng/mL SDF-1 + 20% Yiguanjian drug serum. YGJ: MSCs were cultured in medium supplemented with 20% Yiguanjian drug-containing serum. Data are shown as mean  $\pm$  standard deviation (SD) from 5 independent experiments. <sup>\*</sup> $P < 0.05$  HGF + SDF-1 + Yiguanjian drug serum versus HGF + SDF-1 + normal serum at the same time. <sup>△</sup> $P < 0.05$ : 28 days versus 7 days under the same induction condition. <sup>#</sup> $P < 0.05$ : 14 days versus 7 days and 21 days versus 14 days under the same induction condition.

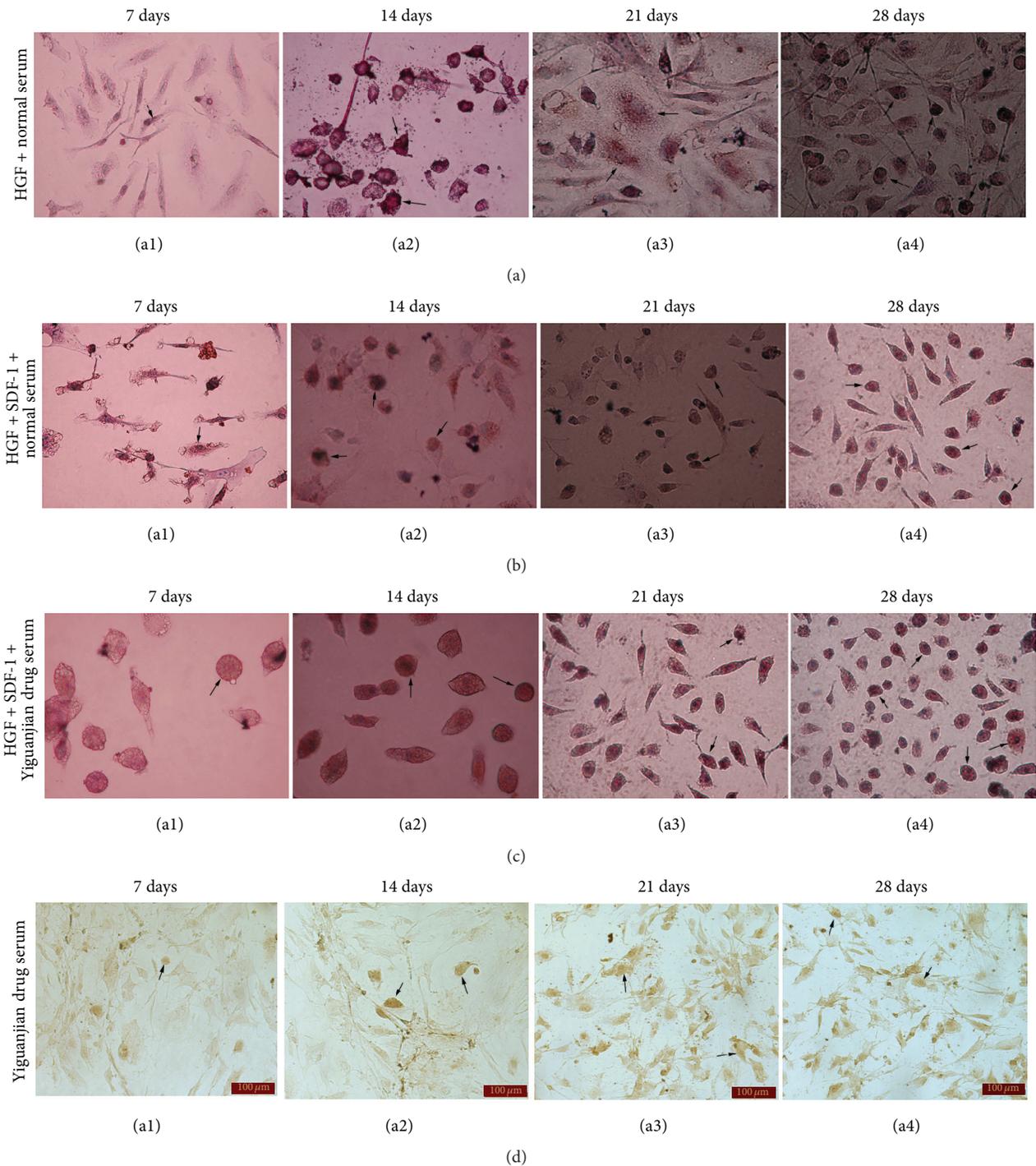


FIGURE 4: Albumin (ALB) expression in differentiated BM-derived MSCs (P2). MSCs were cultured in the presence of 20 ng/mL HGF + 15% normal serum (a), 20 ng/mL HGF + 50 ng/mL SDF-1 + 15% normal serum (b), 20 ng/mL HGF + 50 ng/mL SDF-1 + 20% Yiguanjian decoction (c), or 20% Yiguanjian drug-containing serum (d), and ALB was visualized by ICC at the indicated time points (magnification  $\times 40$ : (a): (a3)-(a4) and (c): (a1)-(a2); magnification  $\times 20$ : (a): (a1)-(a2), (b, c): (a3)-(a4), and (d)). ALB positive staining was observed at day 14 of culture in HGF + normal serum-treated BM-derived MSCs, and ALB staining density increased until day 28 (a). ALB staining was observed at day 7 of culture in HGF + SDF-1 + normal serum-treated BM-derived MSCs, and ALB staining density increased until day 28 (b). ALB staining was observed at day 7 of culture in HGF + SDF-1 + Yiguanjian decoction-treated BM-derived MSCs, and ALB staining density increased until day 28 (c). ALB positive staining cells in YGJ group could be observed at day 14 after induction, and increased gradually to day 21, and there was a reduction at day 28 (d).

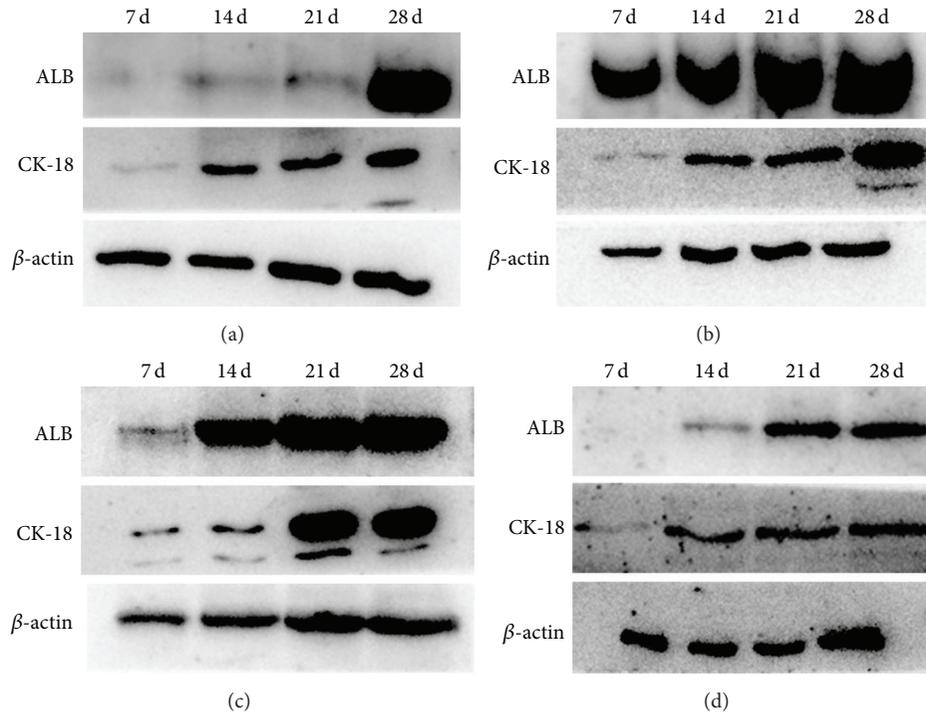


FIGURE 5: ALB and CK-18 protein expressions in differentiated BM-derived MSCs (P2). MSCs were cultured in the presence of 20 ng/mL HGF + 15% normal serum (a), 20 ng/mL HGF + 50 ng/mL SDF-1 + 15% normal serum (b), 20 ng/mL HGF + 50 ng/mL SDF-1 + 20% Yiguanjian drug-containing serum (c), or 20% Yiguanjian drug-containing serum (d), and protein expression was determined by western blot.  $\beta$ -actin was used as an inner control.

TABLE 2: Comparison of ALB and CK-18 expression of induced BM-MSCs by western blot.

Group		7 days	14 days	21 days	28 days
HGF + normal serum	ALB	$0.33 \pm 0.099$	$0.435 \pm 0.064$	$0.51 \pm 0.198$	$2.515 \pm 0.375^{**}$
	CK-18	$0.113 \pm 0.064$	$0.443 \pm 0.28$	$0.477 \pm 0.294$	$1.087 \pm 0.482^{**}$
HGF + SDF-1 + normal serum	ALB	$3.004 \pm 0.142^{**}$	$4.346 \pm 0.049^{**}$	$7.714 \pm 0.073^{**}$	$11.257 \pm 3.425^{**}$
	CK-18	$0.099 \pm 0.007$	$0.23 \pm 0.013$	$0.238 \pm 0.001$	$0.562 \pm 0.21^*$
HGF + SDF-1 + YGJ	ALB	$0.769 \pm 0.04^{**}$	$2.286 \pm 0.038^{**}$	$2.411 \pm 0.002^{**}$	$2.826 \pm 0.014^{**}$
	CK-18	$0.251 \pm 0.167$	$0.387 \pm 0.138$	$2.281 \pm 0.741$	$2.712 \pm 0.537$
YGJ	ALB	$0.452 \pm 0.04$	$1.145 \pm 0.09^{\#}$	$2.143 \pm 0.088$	$1.685 \pm 0.357$
	CK-18	$0.312 \pm 0.041$	$0.963 \pm 0.145^{\#}$	$1.283 \pm 0.104^{\#}$	$1.119 \pm 0.198$

The data are shown as mean  $\pm$  SD. \* $P < 0.05$ , \*\* $P < 0.01$  compared with other times in the same induction group.  $^{\#}P < 0.05$ : 14 days versus 7 days and 21 days versus 14 days.

**3.4. CK-18 Protein Level during Differentiation of BM-Derived MSCs.** Immunocytochemical staining for CK-18 was observed at day 14 in cells cultured with HGF and normal serum, and positive staining intensity increased until day 28 (Figure 6(a)). CK-18 positive staining was observed earlier in cells cultured with HGF, SDF-1, and normal serum or those cultured with HGF, SDF-1, and Yiguanjian drug-containing serum, and staining intensity increased from day 7 until day 28 (Figures 6(b) and 6(c)). Positive staining for CK-18 was observed at day 14 in cells cultured with YGJ, and staining intensity increased until day 28 (Figure 6(d)). Cultures supplemented with HGF, SDF-1, and YGJ contained significantly higher proportion of CK-18 positive cells than cultures supplemented with HGF, SDF-1, and normal serum

at day 7 ( $45.76 \pm 2.20\%$  versus  $34.00 \pm 1.85\%$ ) ( $P < 0.05$ ), indicating that CK-18 expression appeared more rapidly in the former cultures (Table 3). However, by day 14 onward, there was no significant difference in the fraction of CK-18 positive cells in cultures supplemented with HGF, SDF-1, and normal serum or HGF, SDF-1, and YGJ (Table 3).

These observations corroborated the results of western blot quantification. CK-18 protein expression in cultures supplemented with HGF at the 28th day had a significant difference compared with the other time (all  $P < 0.01$ ) (Table 2 and Figure 5(a)). CK-18 protein expression in HGF + SDF-1 + normal serum at the 28th day had a significant difference compared with the other time (all  $P < 0.05$ ) (Table 2 and Figure 5(b)). CK-18 protein expression in

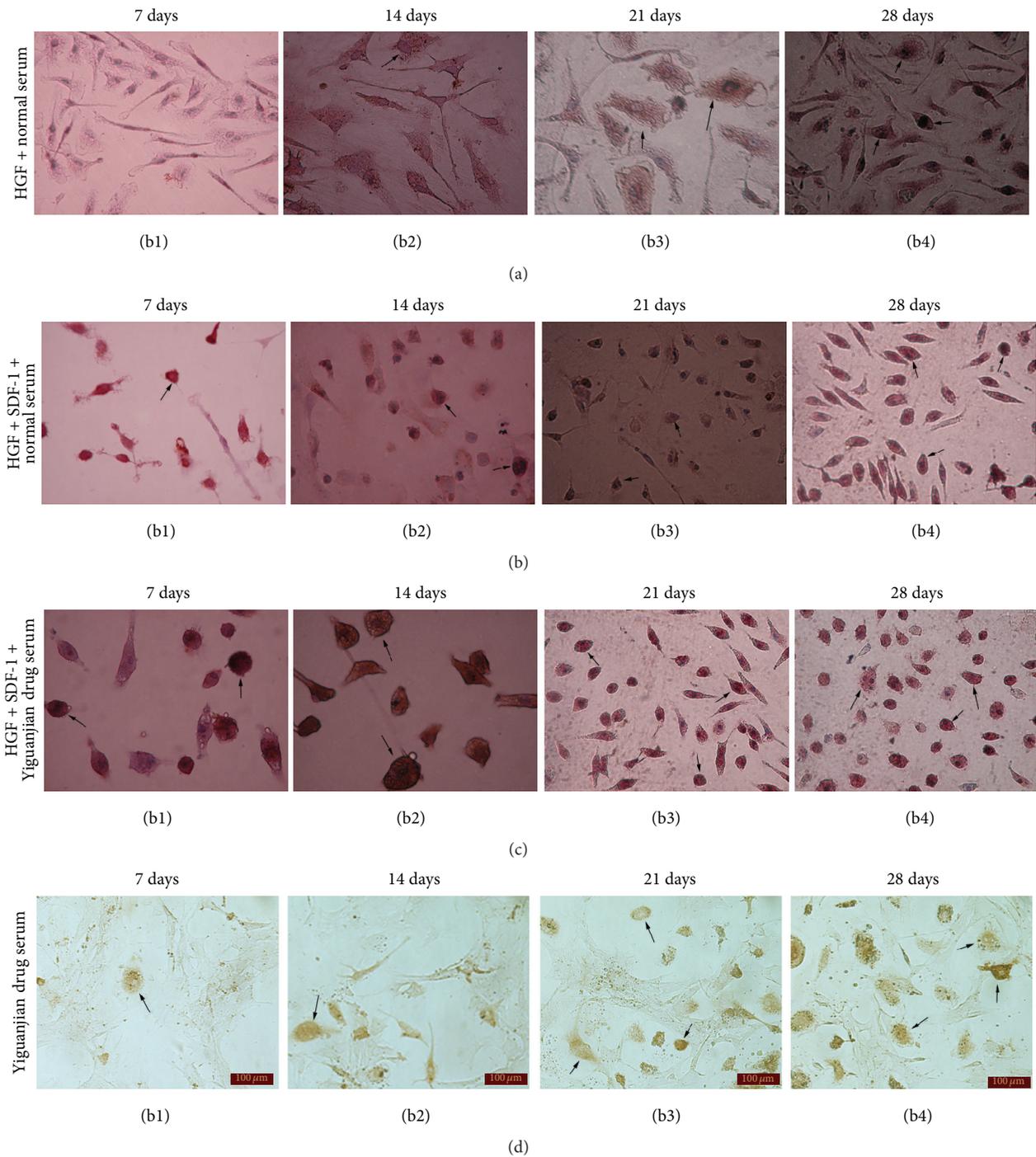


FIGURE 6: Cytokeratin-18 (CK-18) expression in differentiated BM-derived MSCs (P2). BM-MSCs were cultured in the presence of 20 ng/mL HGF + 15% normal serum (a), 20 ng/mL HGF + 50 ng/mL SDF-1 + 15% normal serum (b), 20 ng/mL HGF + 50 ng/mL SDF-1 + 20% YGJ (c), or 20% YGJ (d), and CK-18 was visualized by ICC at the indicated time points (magnification  $\times 40$ : (a): (b2)-(b3) and (c): (b1)-(b2); magnification  $\times 20$ : (a): (b1) and (b4), (b, c): (b3)-(b4), and (d)). CK-18 staining was observed at day 14 of culture in HGF + normal serum-treated BM-MSCs, and CK-18 staining density increased until day 28 (a). CK-18 staining was observed at day 7 of culture in HGF + SDF-1 + normal serum-treated BM-MSCs, and CK-18 staining density increased until day 28 (b). CK-18 staining was observed at day 7 of culture in HGF + SDF-1 + YGJ-treated BM-MSCs, and CK-18 staining density increased until day 28 (c). Positive staining for CK-18 was observed at day 14 in cells cultured with YGJ, and staining intensity increased until day 28 (d).

TABLE 3: Cytokeratin-18 (CK-18) in P2 BM-derived MSCs determined by ICC.

	CK-18 <sup>+</sup> BM-derived MSCs (%)			
	7 days	14 days	21 days	28 days
Negative controls	0	0	0	0
HGF + normal serum	0	42.19 ± 3.78	47.46 ± 2.75	68.61 ± 1.23 <sup>△</sup>
HGF + SDF-1 + normal serum	34.00 ± 1.85	47.68 ± 1.75	50.55 ± 1.01	72.45 ± 4.01 <sup>△</sup>
HGF + SDF-1 + YGJ	45.76 ± 2.20*	56.24 ± 3.51	71.36 ± 1.02	76.72 ± 2.18 <sup>△</sup>
YGJ	4.05 ± 1.28	11.25 ± 2.31 <sup>#</sup>	18.92 ± 1.89 <sup>#</sup>	21.21 ± 3.27

Note. Data are shown as mean ± SD from 5 independent experiments. \*  $P < 0.05$  HGF + SDF-1 + Yiguanjian drug serum versus HGF + SDF-1 + normal serum at the same time point. <sup>△</sup> $P < 0.05$ : 28 days versus 7 days under the same induction condition. <sup>#</sup> $P < 0.05$ : 14 days versus 7 days and 21 days versus 14 days.

HGF + SDF-1 + YGJ-treated cells at the 21st day was significantly higher than those at 7th and 14th days (both  $P < 0.01$ ); furthermore, CK-18 protein expression at the 28th day was higher than those at the 7th and 14th days (both  $P < 0.001$ ) (Table 2 and Figure 5(c)). CK-18 protein expression in YGJ-treated cells increased from the 14th day to the 21st day ( $P < 0.05$ ) (Table 2 and Figure 5(d)).

#### 4. Discussion

We sought to determine whether supplementing cultures with Yiguanjian drug-containing serum could enhance isolated murine BM-MSCs' differentiation towards hepatocytes and cholangiocytes. We prepared the Yiguanjian drug-containing serum by giving mice Yiguanjian decoction by gavage for 3 days and obtained their blood to prepare the drug-containing serum. We isolated MSCs from murine bone marrow by adhesion. Differentiation towards hepatocytes and cholangiocytes was induced by supplementing culture medium with 20 ng/mL HGF, 50 ng/mL SDF-1, and/or 20% Yiguanjian drug-containing serum.

Albumin is expressed in the embryonic liver, increasing gradually with the liver maturation, and is one of the most commonly used reliable indicators of mature hepatocytes. It is mainly secreted and synthesized by hepatocytes [33]. As a specific marker of biliary epithelial cells, CK-18 is not expressed in infantile hepatic progenitor cells but is a relatively specific marker of mature hepatocytes [33]. So we detected a hepatocyte phenotype by staining for albumin and a cholangiocyte phenotype by staining for CK-18. We found that medium added to HGF + SDF-1 or Yiguanjian drug-containing serum alone could induce BM-MSCs' hepatic differentiation as we detected the expression of ALB and CK-18 in group HGF + SDF-1 and group YGJ in addition to cells' morphological changes during induction, and supplementation with YGJ induced differentiation more rapidly than using HGF and SDF-1 alone, although after 28 days in culture there was no significant difference in the fraction of cells positively expressing ALB or CK-18 between the cultures supplemented with HGF + SDF-1 and those supplemented with HGF + SDF-1 + YGJ.

These findings suggest that HGF + SDF-1 or Yiguanjian drug-containing serum alone could induce BM-MSCs' differentiation to hepatocytes and cholangiocytes, and Yiguanjian has a synergistic effect with SDF-1 and HGF as it could enhance the process of differentiation.

HGF binding its receptor HGFR would increase the interaction between the cytokine receptor and ligand and accelerate stem cell mitosis, strengthening differentiation of stem cells into hepatic cells. MSCs can express CXCR4 and c-met cytokine receptor; when exogenous SDF-1 and HGF are added to the cell culture, the MAPK pathway is stimulated [34–36]. Activation of protein kinase MSK1 enhances ALB and CK-18 gene promoter phosphorylation and epigenetic modification can induce multidirectional differentiation. Addition of Yiguanjian drug-containing serum enhanced this effect, and as CXCR4 is the specific receptor of SDF-1, we speculated that YGJ induces BM-MSCs' hepatic differentiation via SDF-1/CXCR4 pathway. However further work will be required to determine the mechanism of Yiguanjian action and explore the active substances of Yiguanjian through performing the chemical analysis in this formula.

#### 5. Conclusions

In conclusion, we found that addition of Yiguanjian drug-containing serum can enhance the speed to induce differentiation of murine BM-derived MSCs into hepatocytes by supplementing HGF and SDF-1 *in vitro*. Yiguanjian might promote liver cell maturation, protect mature hepatocytes, or inhibit hepatic apoptosis. However, these findings in this study are preliminary, and the special mechanism of Yiguanjian and the properties of these *ex vivo* expanded differentiated cells in transplant experiments on animals will need to be further investigated as well.

#### Competing Interests

The authors declare that they have no competing interests.

#### Acknowledgments

This work was supported by the National Natural Science Foundation of China (nos. 81273925 and 81573751). The authors wish to sincerely thank Professor Zhu from the First Affiliated Hospital of Dalian Medical University for her thoughtful comments. They also sincerely thank all the staff in Center Laboratory and Pathology Department of the First Affiliated Hospital of Dalian Medical University for support.

## References

- [1] J. P. Iredale, "Cirrhosis, new research provides a basis for rational and targeted treatments," *The British Medical Journal*, vol. 327, no. 7407, pp. 143–147, 2003.
- [2] D. S. Lee, W. H. Gil, H. H. Lee et al., "Factors affecting graft survival after living donor liver transplantation," *Transplantation Proceedings*, vol. 36, no. 8, pp. 2255–2256, 2004.
- [3] M.-E. M. Amer, S. Z. El-Sayed, W. A. El-Kheir et al., "Clinical and laboratory evaluation of patients with end-stage liver cell failure injected with bone marrow-derived hepatocyte-like cells," *European Journal of Gastroenterology and Hepatology*, vol. 23, no. 10, pp. 936–941, 2011.
- [4] M. El-Ansary, I. Abdel-Aziz, S. Mogawer et al., "Phase II trial: undifferentiated versus differentiated autologous mesenchymal stem cells transplantation in Egyptian patients with HCV induced liver cirrhosis," *Stem Cell Reviews and Reports*, vol. 8, no. 3, pp. 972–981, 2012.
- [5] Y. O. Jang, Y. J. Kim, S. K. Baik et al., "Histological improvement following administration of autologous bone marrow-derived mesenchymal stem cells for alcoholic cirrhosis: a pilot study," *Liver International*, vol. 34, no. 1, pp. 33–41, 2014.
- [6] P. Kharaziha, P. M. Hellström, B. Noorinayer et al., "Improvement of liver function in liver cirrhosis patients after autologous mesenchymal stem cell injection: a phase I-II clinical trial," *European Journal of Gastroenterology and Hepatology*, vol. 21, no. 10, pp. 1199–1205, 2009.
- [7] M. Mohamadnejad, K. Alimoghaddam, M. Mohyeddin-Bonab et al., "Phase 1 trial of autologous bone marrow mesenchymal stem cell transplantation in patients with decompensated liver cirrhosis," *Archives of Iranian Medicine*, vol. 10, no. 4, pp. 459–466, 2007.
- [8] M. Mohamadnejad, M. Namiri, M. Bagheri et al., "Phase 1 human trial of autologous bone marrow-hematopoietic stem cell transplantation in patients with decompensated cirrhosis," *World Journal of Gastroenterology*, vol. 13, no. 24, pp. 3359–3363, 2007.
- [9] M. Mohamadnejad, K. Alimoghaddam, M. Bagheri et al., "Randomized placebo-controlled trial of mesenchymal stem cell transplantation in decompensated cirrhosis," *Liver International*, vol. 33, no. 10, pp. 1490–1496, 2013.
- [10] C.-H. Park, S. H. Bae, H. Y. Kim et al., "A pilot study of autologous CD34-depleted bone marrow mononuclear cell transplantation via the hepatic artery in five patients with liver failure," *Cytotherapy*, vol. 15, no. 12, pp. 1571–1579, 2013.
- [11] S. Terai, T. Ishikawa, K. Omori et al., "Improved liver function in patients with liver cirrhosis after autologous bone marrow cell infusion therapy," *Stem Cells*, vol. 24, no. 10, pp. 2292–2298, 2006.
- [12] L.-J. Dai, H. Y. Li, L.-X. Guan, G. Ritchie, and J. X. Zhou, "The therapeutic potential of bone marrow-derived mesenchymal stem cells on hepatic cirrhosis," *Stem Cell Research*, vol. 2, no. 1, pp. 16–25, 2009.
- [13] X.-Q. Kang, W.-J. Zang, T.-S. Song et al., "Rat bone marrow mesenchymal stem cells differentiate into hepatocytes in vitro," *World Journal of Gastroenterology*, vol. 11, no. 22, pp. 3479–3484, 2005.
- [14] X.-L. Shi, Y.-D. Qiu, X.-Y. Wu et al., "In vitro differentiation of mouse bone marrow mononuclear cells into hepatocyte-like cells," *Hepatology Research*, vol. 31, no. 4, pp. 223–231, 2005.
- [15] N. Lin, J. Lin, L. Bo, P. Weidong, S. Chen, and R. Xu, "Differentiation of bone marrow-derived mesenchymal stem cells into hepatocyte-like cells in an alginate scaffold," *Cell Proliferation*, vol. 43, no. 5, pp. 427–434, 2010.
- [16] S. Oyagi, M. Hirose, M. Kojima et al., "Therapeutic effect of transplanting HGF-treated bone marrow mesenchymal cells into CCl<sub>4</sub>-injured rats," *Journal of Hepatology*, vol. 44, no. 4, pp. 742–748, 2006.
- [17] S. Snykers, J. De Kock, V. Tamara, and V. Rogiers, "Hepatic differentiation of mesenchymal stem cells: in vitro strategies," *Methods in Molecular Biology*, vol. 698, pp. 305–314, 2011.
- [18] P. P. Wang, J. H. Wang, Z. P. Yan et al., "Expression of hepatocyte-like phenotypes in bone marrow stromal cells after HGF induction," *Biochemical and Biophysical Research Communications*, vol. 320, no. 3, pp. 712–716, 2004.
- [19] S. Bhakta, P. Hong, and O. Koc, "The surface adhesion molecule CXCR4 stimulates mesenchymal stem cell migration to stromal cell-derived factor-1 in vitro but does not decrease apoptosis under serum deprivation," *Cardiovascular Revascularization Medicine*, vol. 7, no. 1, pp. 19–24, 2006.
- [20] Y. Matsuda-Hashii, K. Takai, H. Ohta et al., "Hepatocyte growth factor plays roles in the induction and autocrine maintenance of bone marrow stromal cell IL-11, SDF-1 $\alpha$ , and stem cell factor," *Experimental Hematology*, vol. 32, no. 10, pp. 955–961, 2004.
- [21] I. Petit, D. Jin, and S. Rafii, "The SDF-1-CXCR4 signaling pathway: a molecular hub modulating neo-angiogenesis," *Trends in Immunology*, vol. 28, no. 7, pp. 299–307, 2007.
- [22] W. Chen, J.-Y. Chen, Y.-T. Tung et al., "High-frequency ultrasound imaging to evaluate liver fibrosis progression in rats and yi guan jian herbal therapeutic effects," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 302325, 11 pages, 2013.
- [23] X. Gou, Q. Tao, Q. Feng et al., "Urine metabolic profile changes of CCl<sub>4</sub>-liver fibrosis in rats and intervention effects of Yi Guan Jian Decoction using metabonomic approach," *BMC Complementary and Alternative Medicine*, vol. 13, article 123, 2013.
- [24] H.-J. Lin, J.-Y. Chen, C.-F. Lin et al., "Hepatoprotective effects of Yi Guan Jian, an herbal medicine, in rats with dimethylnitrosamine-induced liver fibrosis," *Journal of Ethnopharmacology*, vol. 134, no. 3, pp. 953–960, 2011.
- [25] H. J. Lin, C. P. Tseng, C. F. Lin et al., "A Chinese herbal decoction, modified Yi Guan Jian, induces apoptosis in hepatic stellate cells through an ROS-mediated mitochondrial/caspase pathway," *Evidence-Based Complementary and Alternative Medicine*, vol. 2011, Article ID 459531, 8 pages, 2011.
- [26] X.-L. Wang, D.-W. Jia, H.-Y. Liu et al., "Effect of Yiguanjian decoction on cell differentiation and proliferation in CCl<sub>4</sub>-treated mice," *World Journal of Gastroenterology*, vol. 18, no. 25, pp. 3235–3249, 2012.
- [27] A. J. Friedenstein, R. K. Chailakhyan, and U. V. Gerasimov, "Bone marrow osteogenic stem cells: in vitro cultivation and transplantation in diffusion chambers," *Cell and Tissue Kinetics*, vol. 20, no. 3, pp. 263–272, 1987.
- [28] H. Zhu, Z.-K. Guo, X.-X. Jiang et al., "A protocol for isolation and culture of mesenchymal stem cells from mouse compact bone," *Nature Protocols*, vol. 5, no. 3, pp. 550–560, 2010.
- [29] Y. Kuang, "Regulation of different concentrations of hepatocyte growth factor and fibroblast growth factor on rat liver stem cell proliferation," *International Journal of Pathology and Clinical Medicine*, vol. 30, pp. 106–109, 2010.
- [30] L. Zeng, L. Yang, and J. Yuan, "Experimental study of HGF, EGF effect on proliferation of cultured rat hepatocytes in vitro,"

*Laboratory Animal and Comparative Medicine*, vol. 29, pp. 361–364, 2009.

- [31] P. Ding, Z. Feng, and Z. Yang, “In vitro study of the migration of marrow stromal cells induced by SDF-1,” *Chinese Journal of Neuromedicine*, vol. 6, pp. 225–227, 2007.
- [32] D. Kong, N. Gao, and Y. Zhang, “Migration experiment in vitro of bone marrow mesenchymal stem cells by CXCR4 gene modification,” *Journal of Biomedical Engineering*, vol. 26, pp. 595–600, 2009.
- [33] C. Lange, P. Bassler, M. V. Lioznov et al., “Liver-specific gene expression in mesenchymal stem cells is induced by liver cells,” *World Journal of Gastroenterology*, vol. 11, no. 29, pp. 4497–4504, 2005.
- [34] J. Li, Z. Zhao, J. Liu et al., “MEK/ERK and p38 MAPK regulate chondrogenesis of rat bone marrow mesenchymal stem cells through delicate interaction with TGF- $\beta$ 1/Smads pathway,” *Cell Proliferation*, vol. 43, no. 4, pp. 333–343, 2010.
- [35] S. Peng, G. Zhou, K. D. K. Luk et al., “Strontium promotes osteogenic differentiation of mesenchymal stem cells through the Ras/MAPK signaling pathway,” *Cellular Physiology and Biochemistry*, vol. 23, no. 1–3, pp. 165–174, 2009.
- [36] S. Zhou, S. Lechpammer, J. S. Greenberger, and J. Glowacki, “Hypoxia inhibition of adipocytogenesis in human bone marrow stromal cells requires transforming growth factor- $\beta$ /Smad3 signaling,” *Journal of Biological Chemistry*, vol. 280, no. 24, pp. 22688–22696, 2005.